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Research Note

Detection of Zearalenone and Related Metabolites by Fluorescence Polarization Immunoassay

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ABSTRACT

Zearalenone is an estrogenic mycotoxin commonly found in grains throughout the world. A number of instrument- and antibody-based methods including enzyme-linked immunosorbent assays (ELISAs) have been developed to detect zearalenone (ZEN) and related toxins in commodities and foods. Although convenient, the commercial ELISAs for small molecules such as ZEN require a washing step to separate bound and unbound enzyme label before detection. In fluorescence polarization immunoassays, separation of bound and unbound label is not required, a property that reduces the time needed to perform the assays. We developed a fluorescence polarization immunoassay for ZEN in maize. When combined with a rapid extraction technique, the assay could be used to detect as little as 0.11 μg of ZEN g^{-1} maize within 10 min. The assay showed cross-reactivity to the ZEN analogs zearalanone, α-zearalanol, α-zearalenol, β-zearalenol, and β-zearalanol of 195, 139, 102, 71, and 20%, respectively, relative to ZEN (100%). Recovery of ZEN from spiked maize over the range of 0.5 to 5 μg g^{-1} averaged 100.2% (n = 12). The fluorescence polarization immunoassay results were comparable to those obtained with a liquid chromatographic method for the analysis of 60 naturally contaminated maize samples and maize samples amended with culture material. The fluorescence polarization immunoassay provides a rapid method for screening of maize for ZEN.

Zearalenone (ZEN) is a mycotoxin produced by several fungi, in particular *Fusarium graminearum* (sexual state *Gibberella zeae*). The fungi and their mycotoxins are frequent contaminants of maize and barley but are also found in wheat, oats, rye, and sorghum. Surveys have indicated that ZEN is routinely present in maize throughout the world *(17)*. The acute toxicity of ZEN is relatively low *(4)*. However, ZEN and related compounds, in particular α-zearalenol (α-ZEOL) and β-zearalenol (β-ZEOL), are nonsteroidal compounds (Fig. 1) with potent estrogenic activity. An analog of ZEN, α-zearalanol (zeranol or α-ZAOL), has anabolic effects and is used in some countries as a growth promoter in cattle. Although the U.S. Food and Drug Administration has not established official regulatory or advisory levels for ZEN in human food, several countries have established guidelines or tolerances that range from 0.03 to 1 μg g^{-1} *(17)*.

Radioimmunoassays and enzyme-linked immunosorbent assays (ELISAs) for detection of ZEN have been developed *(2, 6, 11, 14–16)*. A ZEN ELISA is available commercially and has been collaboratively studied *(1)*. Both polyclonal and monoclonal anti-ZEN antibodies are available commercially (Sigma, St. Louis, Mo.). In addition to ELISAs, a dipstick immunoassay for several mycotoxins in wheat, including ZEN, has been developed *(12)*.

Although several of the existing ZEN ELISAs are very sensitive, they require a washing step to separate unbound toxin-enzyme conjugate from the solid phase. Elimination of this washing step would increase the speed and ease of use of immunoassays. Fluorescence polarization (FP) is a technology that has been used in medical and veterinary diagnostics to detect small molecules in solution without the need for a washing step. FP is minimally affected by solution opacity or color, which affect the intensity more than the orientation of the fluorescence. The FP immunoassay utilizes the interaction of a toxin-specific antibody with a toxin-fluorophore conjugate (tracer) to effectively decrease the rate of rotation of the tracer. Binding of the antibody to the tracer increases polarization. In the presence of free toxin, less of the antibody is bound to the tracer, reducing polarization.

The objective of the present work was to extend this FP technology to the detection of ZEN and its analogs in maize.

MATERIALS AND METHODS

Reagents. Except where noted otherwise, deionized water (Nanopure II, Sybron/Barnstead, Boston, Mass.) was used in the preparation of all reagents. All solvents were high-pressure liquid chromatography (HPLC) grade. ZEN, α-ZEOL, β-ZEOL, and α-ZAOL were obtained from International Minerals & Chemicals Corporation (Terre Haute, Ind.), zearalanone (ZAN) and β-zearalanol (taleranol) were purchased from Sigma, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) was purchased from Aldrich (Milwaukee, Wis.), *N*-hydroxysuccinimide was purchased from Pierce Chemical Company (Rockford, Ill.), and 4′-(aminomethyl) fluorescein hydrochloride (4′-AMF) was

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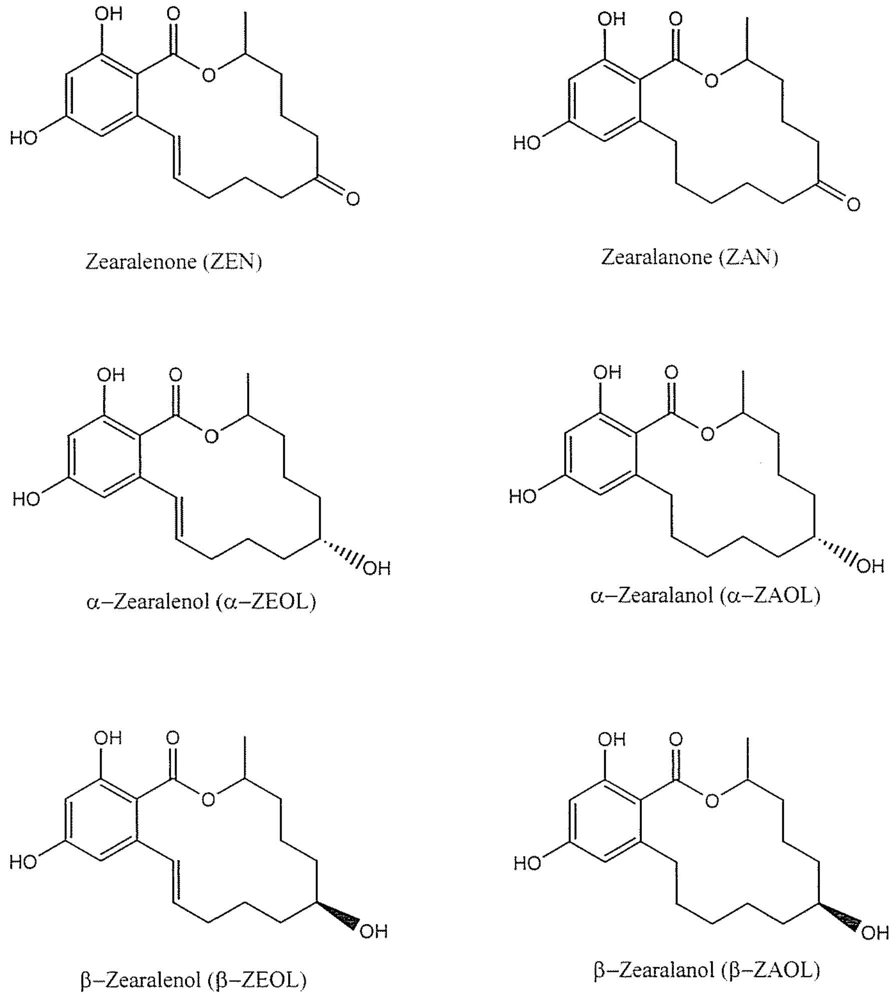
FIGURE 1. *Structure of zearalenone and related analogs.*



purchased from Molecular Probes (Eugene, Oreg.). ZEN antibody (monoclonal, IgE isotype) was derived from a mouse immunized with a ZEN-KLH conjugate and was produced by the ZER-70 hybridoma cell line (product Z-1751, Sigma).

Preparation of ZEN-FL tracer. The 6′-carboxymethyloxime of ZEN was prepared according to the method of Thouvenot and Morfin *(15)* and reacted with 4′-AMF to produce a ZEN-fluorescein conjugate (ZEN-FL). Ten milligrams of 6′-carboxymethyloxime of ZEN in 1 ml of methanol was mixed with 1 ml of 0.1 M morpholineethanesulfonic acid buffer (pH 5.0), 30 mg of EDC, and then 50 mg of *N*-hydroxysuccinimide. This mixture was held at ambient temperature for 15 min, and 10 mg of 4′-AMF (in 1 ml *N,N*-dimethylformamide) was added followed by 1 ml of 0.1 M sodium borate (pH 9.3). This mixture was held at ambient temperature with stirring for 3 h, after which another 1 ml of sodium borate solution was added. The mixture was held for an additional 3 h at ambient temperature and a further 21 h at 4°C. The reaction was diluted with 25 ml of 0.01 M sodium bicarbonate buffer (pH 7.7) and extracted four times with 50 ml of ethylacetate. The organic phase was dried under vacuum evaporation, reconstituted with methanol, loaded onto a C18 solid phase extraction column (Mega BondElut, Varian, Palo Alto, Calif.), and washed with water-methanol mixtures, and the ZEN-FL was eluted with methanol-water (4:1, vol/vol).

Extraction of samples. Samples of whole kernel corn were ground fine enough to pass a #20 sieve. A 25-g sample of the ground corn was mixed with 125 ml of acetonitrile-water (84:16 vol/vol) and blended for 3 min (Waring Product Division, New Hartford, Conn.). The blended material was poured over a #2V filter (Whatman International Ltd., Maidstone, UK), and the filtered extract was collected and used directly for testing by FP immunoassay or HPLC. Spiking and recovery studies were conducted using maize that contained <0.025 μg of ZEN g^{-1} as determined by HPLC. For these studies, the maize was spiked with small volumes of a 100 μg ml^{-1} stock solution of ZEN in acetonitrile-water, mixed, and allowed to dry for 24 to 72 h at ambient temperature before extraction.

Additional comparisons were conducted with 21 naturally contaminated maize samples and 39 mixed (amended) samples of maize. The ZEN culture material was produced using the *F. graminearum* isolate "Mapleton 10" *(9)* (graciously provided by Dr. Chester Mirocha, University of Minnesota, Minneapolis). The amended samples were prepared by mixing small amounts of culture material with maize from a variety of sources.

HPLC assay of maize. Acetonitrile-water extracts of maize were tested by reverse-phase HPLC without further cleanup. An HPLC method for ZEN in maize that uses fluorescence detection and does not require a cleanup step has been reported *(5)*. Samples assayed as having <1 μg of ZEN g^{-1} were retested using an immunoaffinity column cleanup procedure. For these samples, 4 ml of extract was mixed with 10 ml of phosphate-buffered saline (PBS: 0.01 M sodium phosphate, 0.15 M sodium chloride, and 0.003 M sodium azide in water, pH 7.2) and passed through the immunoaffinity column (ZearalaTest, Vicam, Watertown, Mass.)

at a flow rate of 1 drop s^{-1}. The column was washed with 5 ml of PBS, and the toxin was eluted with 3 ml of methanol. Purified extracts were dried under a gentle stream of nitrogen at 55°C, and the residue was dissolved in 200 μl of acetonitrile-water for injection. The HPLC system consisted of a model P4000 pump (Spectra-Physics, Mountain View, Calif.), a model 7125 injector with a 20-μl sample loop (Rheodyne, Cotati, Calif.), a model FL2000 fluorescence detector (274 nm excitation, 440 nm emission; Spectra-Physics), and a model UV2000 absorbance detector (monitoring at 236 nm; Spectra-Physics) placed in series. For separation, a 5-μm column (inside diameter 150 by 4.6 mm, Zorbax, Hewlett Packard, Chadds Ford, Pa.) with a NewGuard RP-18 7-μm guard column (inside diameter 15 by 3 mm, Applied Biosystems, Foster City, Calif.) was held at 27°C in a model SSI 505 LC column oven (Scientific Systems, Inc., State College, Pa.). The mobile phase was methanol-water (7:3, vol/vol) at a flow rate of 0.5 ml min^{-1}. Under these conditions, retention times were 5.8 min (β-ZAOL), 6.3 min (β-ZEOL), 7.8 min (α-ZAOL), 8.3 min (α-ZEOL), 9.3 min (ZAN), and 10.2 min (ZEN). Data were collected simultaneously using Allchrom chromatography data system software (Alltech Associates, Inc., Deerfield, Ill.). Although both fluorescence and absorbance data were collected, the fluorescence data were used for quantitation of ZEN. The detection limit for ZEN, defined as a signal-to-noise ratio of 3, was 2 ng, which correponds to 25 ng of ZEN g^{-1} of maize or 500 ng of ZEN g^{-1} of maize with or without immunoaffinity column cleanup, respectively.

FP immunoassay. The FP instrument used was a Sentry FP portable unit (Diachemix Corporation, Grayslake, Ill.) described previously *(7)*. ZEN standards were prepared by diluting the ZEN stock solution (100 μg ml^{-1}) with acetonitrile-water (84:16, vol/vol). Antibody working solution was prepared by diluting ZEN monoclonal antibody 1:250 in PBS containing 0.1% ovalbumin. ZEN-FL working solution was prepared fresh daily by diluting the stock solution 1:10,000 in PBS. The tracer intensity was monitored daily to ensure an optimum intensity of 350,000 to 500,000.

Glass culture tubes (10 by 75 mm, VWR Scientific, West Chester, Pa.) were used as cuvettes for the assays. PBS (0.9 ml) was added to each tube followed by 0.1 ml of the antibody working solution and 20 μl of the sample or standard. After thorough mixing, the test solution was placed in the instrument and used as the blank. Tracer (20 μl of the ZEN-FL working solution) was then added and mixed. The test solution containing tracer was then returned to the fluorometer, and the signal (mP) was measured. For experiments to elucidate the kinetics of the reaction, measurements were made after holding at ambient temperature for times ranging from 30 s to 15 min. In all other experiments, the holding time was 2 min. ZEN content of maize samples was estimated relative to a logistic dose-response fit of a calibration curve of ZEN in acetonitrile-water (84:16, vol/vol) (TableCurve software, Jandel Scientific, San Rafael, Calif.) tested daily.

RESULTS AND DISCUSSION

Analytical methodology for detection of ZEN and related compounds is well developed, and the ELISAs that have been described are sensitive enough to detect ZEN at levels well below those considered to be hazardous. However, the ELISAs by necessity require the separation of the bound label (toxin-enzyme conjugate attached to antibody or antibody-enzyme conjugate attached to a toxin-protein conjugate) from the unbound label with a washing step. A waiting period is then required for the substrate to react

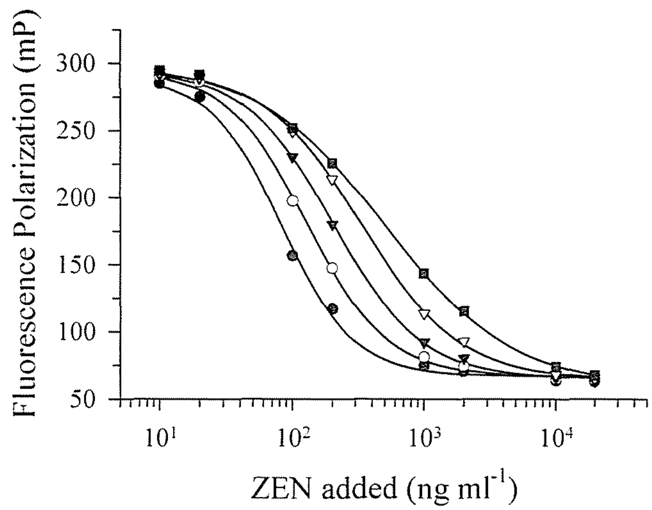


FIGURE 2. *Kinetics of the ZEN-FP immunoassay. Calibration curves were obtained at various times using a tracer incubation of 30 s (●), 1 min (○), 2 min (▼), 5 min (▽), and 15 min (■).*

with the enzyme. The FP format offers the possibility of reducing the number of steps by eliminating the need to separate the bound and free material. We prepared a ZEN-FL tracer and used a commercially available ZEN antibody to develop an FP immunoassay for ZEN in maize.

Assays were conducted by mixing antibody and sample in a tube, calibrating the instrument using the mixture as the blank, adding the tracer (ZEN-FL), and then holding the sample for a brief period (30 s to 15 min) before measurement of the FP. Our previous experience with FP immunoassays *(7, 8, 10)* has shown that the duration of tracer incubation is an important factor in the performance of the assay. The effect of the duration of tracer incubation is illustrated in Figure 2. Assays were clearly more sensitive with a shorter incubation time. With a 2-min tracer incubation, the limit of detection of the assay (defined as the concentration that was 3 standard deviations from the response of the toxin-free controls) was 22 ng of ZEN ml^{-1} of extract or 0.11 μg ZEN g^{-1} of maize.

Cross-reactivity of the FP immunoassay. Cross-reactivities of ZEN analogs relative to that of ZEN (100%) were 195% (ZAN), 139% (α-ZAOL), 102% (α-ZEOL), 71% (β-ZEOL), and 20% (β-ZAOL) (Fig. 3). In general, the analogs in which the 1′-2′ double bond was reduced had higher cross-reactivity than did those in which the double bond was present. The exception was that the antibody had a greater activity for β-ZEOL than for β-ZAOL. When the 6′ carbonyl was reduced to a hydroxyl, the antibody had greater activity for the α epimers relative to the β epimers.

Contaminated maize samples. Recovery of ZEN added to maize was very good, particularly at levels >0.2 μg g^{-1}. Recovery at 0.2 μg g^{-1} was poor (55.25%), but recovery over the range of 0.5 to 5 μg g^{-1} averaged 100.2 ± 12.4% (Table 1). In addition to maize samples spiked with ZEN, the FP immunoassay and HPLC were also applied to 60 samples of maize either naturally contaminated with ZEN or amended with *F. graminearum* culture ma-

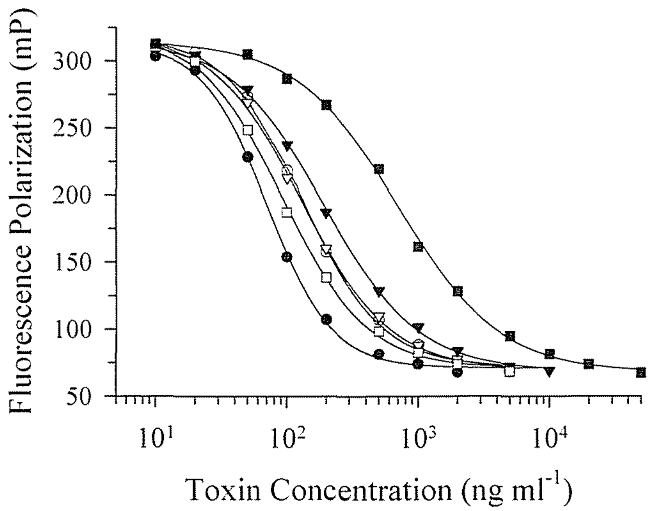


FIGURE 3. *Cross-reactivity of the ZEN-FP immunoassay. The concentrations of toxins at the midpoint of the calibration curves were 70, 99, 134, 137, 192, and 694 ng ml^{-1} for ZAN (●), α-ZAOL (□), α-ZEOL (▽), ZEN (○), β-ZEOL (▼), and β-ZAOL (■), respectively. Data shown are the average of nine replicates, except for ZEN where the data are the average of 18 replicates.*

terial. Of these 60 samples, 21 were naturally contaminated samples that were tested directly without addition of culture material. The remaining 39 samples were prepared from maize to which ZEN-containing culture material was added (amended samples).

For the 21 naturally contaminated maize samples, the FP and HPLC assay results were comparable (Fig. 4A). The linear least squares regression fit was of the form ZEN by FP = 0.10 + 1.02 (ZEN by HPLC), with an r^2 of 0.976. The data for the amended samples were also similar (Fig. 4B), although not as close as those for the naturally contaminated samples: ZEN by FP = 1.02 + 1.39 (ZEN by HPLC), with an r^2 of 0.938.

The difference between the results for the naturally contaminated samples and those for the samples amended with culture material suggested that the culture material was causing a bias in the amended samples. However, the low levels of ZAN, α-ZEOL, α-ZAOL, and β-ZEOL in the culture material, as determined by HPLC, suggest that the contributions of these metabolites to the bias were minimal. The source of the bulk of the bias is unknown. There are other derivatives of ZEN, such as ZEN-glycoside conjugates, that are not detected by most HPLC methods *(3, 13)*, and one of these conjugates may have caused the observed bias. Other FP immunoassays for other mycotoxins also have shown a slight positive bias of 20 to 30% that could not be accounted for based upon known cross-reactivities. The bias is not a limiting constraint upon the use of the FP assays when the screening data are interpreted appropriately. For this reason, we recommend that samples containing large amounts of ZEN, as measured by the FP immunoassay, be tested with an instrument-based method (such as HPLC) to confirm the toxin level.

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TABLE 1. *Recovery of ZEN from spiked maize*

Spiking level (μg g^{-1})	% recovery (mean ± SD)[a]
0.20	55.3 ± 10.3
0.50	82.5 ± 4.0
1.00	96.4 ± 6.1
2.00	110 ± 3.2
5.00	108 ± 10

[a] Average recovery over the range of 0.2 to 5 μg g^{-1} was 91.9 ± 20.6% (n = 16). Average recovery over the range of 0.5 to 5 μg g^{-1} was 100.2 ± 12.4% (n = 12).

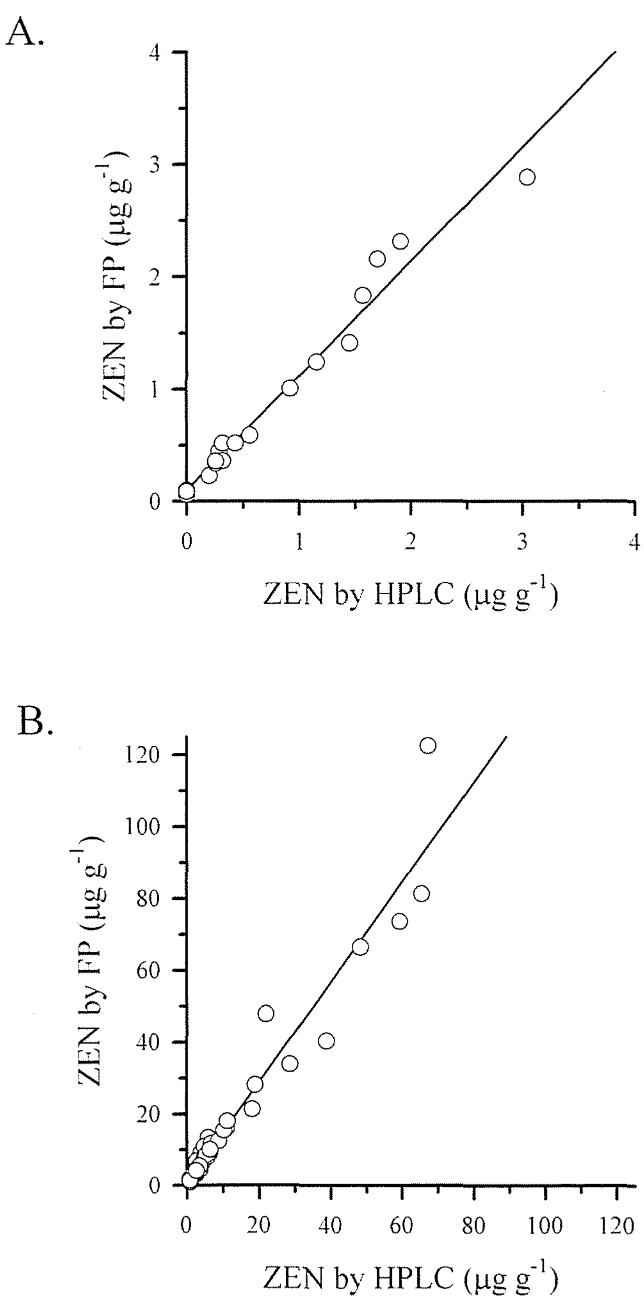

FIGURE 4. *Comparison of ZEN in 60 maize samples analyzed by both HPLC and FP immunoassay. (A) Twenty-one samples of naturally contaminated maize. (B) Thirty-nine samples of various types of maize amended with* F. graminearum *culture material.*

REFERENCES

1. Bennett, G. A., T. C. Nelsen, and B. M. Miller. 1994. Enzyme-linked immunosorbent assay for detection of zearalenone in corn, wheat, and pig feed: collaborative study. *J. AOAC Int.* 77:1500–1509.
2. Dixon, D. E., R. L. Warner, B. P. Ram, L. P. Hart, and J. J. Pestka. 1987. Hybridoma cell line production of a specific monoclonal antibody to the mycotoxins zearalenone and α-zearalenol. *J. Agric. Food Chem.* 35:122–126.
3. Gareis, M., J. Bauer, J. Thiem, G. Plank, S. Grabley, and B. Gedek. 1990. Cleavage of zearalenone-glycoside, a "masked" mycotoxin, during digestion in swine. *J. Vet. Med. B* 37:236–240.
4. Hidy, P. H., R. S. Baldwin, R. L. Greasham, C. L. Keith, and J. R. McMullen. 1977. Zearalenone and some derivatives: production and biological activities. *Adv. Appl. Microbiol.* 22:59–82.
5. Lauren, D. R., and M. A. Ringrose. 1997. Determination of the fate of three *Fusarium* mycotoxins through wet-milling of maize using an improved HPLC analytical technique. *Food Addit. Contam.* 14:435–443.
6. Liu, M.-T., B. P. Ram, L. P. Hart, and J. J. Pestka. 1985. Indirect enzyme-linked immunosorbent assay for the mycotoxin zearalenone. *Appl. Environ. Microbiol.* 50:332–336.
7. Maragos, C. M., M. E. Jolley, R. D. Plattner, and M. Nasir. 2001. Fluorescence polarization as a means for determination of fumonisins in maize. *J. Agric. Food Chem.* 49:596–602.
8. Maragos, C. M., and R. D. Plattner. 2002. Rapid fluorescence polarization immunoassay for the mycotoxin deoxynivalenol in wheat. *J. Agric. Food Chem.* 50:1827–1832.
9. Mirocha, C. J., C. M. Christensen, and G. H. Nelson. 1971. F-2 (zearalenone) estrogenic mycotoxin from *Fusarium*. *Microb. Toxins* 7:107–138.
10. Nasir, M. S., and M. E. Jolley. 2002. Development of a fluorescence polarization assay for the determination of aflatoxins in grains. *J. Agric. Food Chem.* 50:3116–3121.
11. Park, J. W., E. K. Kim, D. H. Shon, and Y. B. Kim. 2002. Occurrence of zearalenone in Korean barley and corn foods. *Food Addit. Contam.* 19:158–162.
12. Schneider, E., E. Usleber, E. Martlbaur, R. Dietrich, and G. Terplan. 1995. Multimycotoxin dipstick enzyme immunoassay applied to wheat. *Food Addit. Contam.* 12:387–393.
13. Schneweis, I., K. Meyer, G. Engelhardt, and J. Bauer. 2002. Occurrence of zearalenone-4-β-D-glucopyranoside in wheat. *J. Agric. Food Chem.* 50:1736–1738.
14. Tanaka, T., R. Teshima, H. Ikebuchi, J. Sawada, and M. Ichinoe. 1995. Sensitive enzyme-linked immunosorbent assay for the mycotoxin zearalenone in barley and Job's-tears. *J. Agric. Food Chem.* 43:946–950.
15. Thouvenot, D., and R. F. Morfin. 1983. Radioimmunoassay for zearalenone and zearalanol in human serum: production, properties, and use of porcine antibodies. *Appl. Environ. Microbiol.* 45:16–23.
16. Towers, N. R., and J. Sprosen. 1992. *Fusarium* mycotoxins in pastoral farming: zearalenone induced infertility in sheep, p. 272–284. *In* P. Gopalakrishnakone and C. K. Tan (ed.), Recent advances in toxinology research, vol. 3. National University of Singapore, Singapore.
17. World Health Organization. 2000. Safety evaluation of certain food additives and contaminants, p. 393–482. *In* WHO Food Additives Series: 44. World Health Organization, Geneva.